ORIGINAL ARTICLE

Unappreciated HLA Antibodies in Adult Immune Thrombocytopenic Purpura

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Background/Purpose: Immune thrombocytopenic purpura (ITP) is an autoimmune disease. Platelet refractoriness is frequently seen in patients with ITP. Platelets express platelet-specific antigens and human leukocyte antigens (HLA). Platelet antibodies to platelet-specific antigens and HLA may be present, but HLA antibodies in patients with ITP have rarely been reported.

Methods: Sera from 44 adult patients with ITP were screened for platelet antibodies by two flow cytometric assays. In method I, platelets from normal donor platelets were used as target cells to screen both platelet-specific antibodies and HLA class I antibodies. In method II, the FlowPRA™ Class I Screening Test kit was used to screen HLA class I antibodies. Fluorescein isothiocyanate (FITC)-conjugated sheep anti-human IgG Fc was used as the staining reagent in both methods. The negative serum control was from one of the normal males with AB blood group who had never received a transfusion. Sera from a pool of five highly sensitized patients were used as the positive control.

Results: Of the 44 sera from patients with ITP, 31 (70.5%) were method I positive, and 28 (63.6%) were method II positive. There was no significant difference between the results of method I and method II ($p = 0.439$). The distribution of the results of these two tests was: both tests positive in 22 sera, method I positive and method II negative in nine sera, method I negative and method II positive in six sera, and both tests negative in seven sera. The mean platelet counts of patients with positive ($41.0 \pm 40.0 \times 10^9$/L) and negative ($40.4 \pm 26.8 \times 10^9$/L) tests by method I did not differ significantly ($p = 0.643$). The mean platelet counts of patients with ($36.7 \pm 31.5 \times 10^9$/L) and without ($48.1 \pm 43.6 \times 10^9$/L) HLA class I antibodies did not differ significantly ($p = 0.59$).

Conclusion: HLA class I antibodies are frequently found in ITP. The screening of platelet antibodies including platelet-specific antibodies and unappreciated HLA class I antibodies is warranted in patients with ITP.
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Key Words: flow cytometry, human leukocyte antigen, immune thrombocytopenic purpura, platelet antibody

Immune thrombocytopenic purpura (ITP) is an autoimmune disease in which platelets are sensitized with antiplatelet autoantibodies and then destroyed by the reticuloendothelial system.[1] Platelet refractoriness is frequently observed in patients with ITP, even in patients without history of transfusion or pregnancy.[2] The factors causing platelet transfusion refractoriness in patients with hemato-oncologic disorders are disseminated intravascular coagulation, concurrent intravenous

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amphotericin B, splenomegaly, human leukocyte antigens (HLA) antibodies, platelet-specific antibody, active bleeding, and fever.[3–7] In patients with ITP, platelet-specific antibodies that recognize platelet glycoproteins have been reported.[8–11] However, HLA antibodies in patients with ITP have rarely been reported.

Flow cytometry is a sensitive method for the detection of platelet antibodies.[12–14] Many platelet antibody tests including flow cytometry have been used in the detection of platelet antibodies in patients with ITP.[15–17] In this study, we used two flow cytometric methods to screen platelet-specific antibodies and/or HLA antibodies in the sera of ITP patients.

Methods

Patients and controls

We studied sera from 44 patients with ITP. The negative serum control was from one of the normal males with AB blood group who had never received a transfusion. Sera from a pool of five highly sensitized patients were used as the positive control.

Method I: flow cytometry using donor platelets as target cells

1. Platelet preparation

We utilized platelets from 10 fresh plateletpheresis components as target cells to screen platelet antibodies (HLA antibodies and/or platelet-specific antibodies) in the sera. Platelets were washed twice with a mixture of phosphate-buffered saline (PBS) and anticoagulant citrate dextrose solution (ACD) formula A (Baxter Healthcare Co., Fenwal Division, Deerfield, IL, USA) (PBS/ACD = 9/1) with pH adjusted to 7.0, by centrifugation at 1400*g* for 5 minutes. Washed platelets were pooled. We adjusted the platelet count to 200×10^9/L with PBS/ACD. The reagent platelets were prepared on the day of analysis and were not stored for future use.

2. Antibody test

Equal volumes (50 μL) of patients' sera and pooled platelets were incubated at 37°C for 30 minutes, respectively. The cells were then washed twice with PBS/ACD at 1400*g* for 5 minutes. The sensitized platelets were resuspended in 100 μL PBS/ACD and incubated for 30 minutes in the dark at room temperature with 10 μL fluorescein isothiocyanate (FITC)-conjugated sheep anti-human IgG Fc (The Binding Site Ltd., Birmingham, UK). They were then washed twice in PBS/ACD. We resuspended the pellet in 200 μL PBS/ACD by shaking the tubes on a vortex for 15 seconds and promptly analyzed using flow cytometry (Epics XL, Coulter Electronics, Hialeah, FL, USA). We analyzed 5000 platelets per test.

A negative reference range was determined by using sera from never-transfused males. We set the analysis region with the negative reference range in the FL1 histogram. Positive results were defined as any test with the percentage of fluorescence exceeding the reference range by 3%.[18] Negative and positive control sera were included in each test.

Method II: flow cytometry using FlowPRA kit

The FlowPRA™ Class I Screening Test (One Lambda Inc., Canoga Park, CA, USA) was used to screen HLA class I antibodies. FlowPRA Class I assays consist of a pool of 30 different bead preparations. Each preparation is coated with HLA class I antigens purified from one of 30 cell lines. All common HLA antigens, as well as many rare HLA antigens, are represented in the pool. Class I-positive and -negative control sera (One Lambda Inc.) were included in each test.

Each serum sample and the negative and positive controls were tested in parallel with FlowPRA Class I beads. A total of 5 μL FlowPRA Class I beads were mixed with 20 μL serum and then incubated at 22°C in the dark for 30 minutes. The beads were then washed twice in 1 mL FlowPRA wash buffer at 9000*g* for 2 minutes. A total of 100 μL of the working FITC-conjugated IgG in the test kit was added to each test and vortexed to mix. Then, it was incubated at 22°C in the dark for 30 minutes. The samples were then washed with 1 mL FlowPRA wash buffer twice, and then resuspended in 0.5 mL of FlowPRA wash buffer. The samples were analyzed using a XL Coulter flow cytometer.

We analyzed 5000 beads per test. Results were recorded as positive when ≥3% of Class I beads exhibited fluorescence above the negative control and/or a distinct second peak at the positive region of the histogram.

Statistical analysis

Statistical analysis of the data was performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). The platelet counts of patients with positive and negative tests were compared using the Mann–Whitney test. The test results of method I and method II were compared using the McNemar test. The relationship between the presence of HLA antibodies and prior transfusion history or pregnancy history was analyzed using the χ^2 test. A p value of less than 0.05 was considered significant.

Results

A total of 44 patients with ITP (28 female, 16 male) were studied. The median age of the ITP patients was 53 years (range, 23–90 years). Their sera were tested for platelet antibodies by methods I and II. The test results and platelet counts are presented in the Table. All assays yielded negative results for negative control sera.

By method I, the 31 serum specimens (70.5%) yielded positive results. HLA class I antibodies were detected in the sera of 28 patients (63.6%) by method II. There was no significant difference ($p = 0.439$) between the results of method I and method II by the McNemar test.

The mean platelet counts of patients with positive ($41.0 \pm 40.0 \times 10^9$/L) and negative ($40.4 \pm 26.8 \times 10^9$/L) tests by method I did not differ significantly ($p = 0.643$). The mean platelet counts of patients with ($36.7 \pm 31.5 \times 10^9$/L) and without ($48.1 \pm 43.6 \times 10^9$/L) HLA class I antibodies did not differ significantly ($p = 0.59$).

Among the 28 patients with HLA class I antibodies, 12 patients had a transfusion history and 18 patients were females. The occurrence of HLA class I antibodies was not significantly correlated with having a transfusion history ($p = 0.333$) or female gender ($p = 1.00$).

Among the 20 female patients with history of pregnancy, 13 (65%) had HLA class I antibodies. The occurrence of HLA class I antibodies in female patients with ITP was not correlated with pregnancy ($p = 1.00$).

Six males and four females without history of transfusion and pregnancy had HLA class I antibodies.

Discussion

ITP is a heterogeneous autoimmune disorder. ITP is due to different immune defects in individual patients. There is evidence of a B-cell disorder, T cell abnormalities and cytokine abnormalities.[19]

In the majority of patients, ITP is caused by an antiplatelet antibody. Viral or bacterial infections may initiate ITP via molecular mimicry or B-cell stimulation.[20–23] Polyclonal activation of B cells may result in autoantibodies being formed.[24]

During B lymphocyte development, antibodies are assembled by random gene segment reassortment to produce a vast number of specificities, and some of the antibodies are self-reactive.

Table. Results of platelet antibody screening and platelet counts in 44 patients with immune thrombocytopenic purpura

Group	Method I	Method II	Number of patients	Platelet count ($\times 10^9$/L)	p*
I	Positive	Positive	22	35.5 ± 34.2	–
II	Positive	Negative	9	54.6 ± 51.4	0.513
III	Negative	Positive	6	41.0 ± 20.7	0.370
IV	Negative	Negative	7	39.9 ± 32.8	0.740

**Comparison of each group with group I using the Mann–Whitney test. Method I = flow cytometry using donor platelets as target cells; Method II = FlowPRA™ Class I screening test.*

Interruption of the checkpoints at which the immature B cells producing autoreactive antibodies are removed from the circulation will result in an increase in the numbers of autoantibodies.[25] Some patients with immune cytopenia had polyclonal autoantibodies against platelets, red cells and granulocytes. The autoantibodies are specific to their target cells and, as shown by absorption and elution, do not cross-react. Some ITP cases may be associated with autoimmune hemolytic anemia or autoimmune neutropenia.[26–28] In our study, HLA class I antibodies were found in six males and four females without both transfusion history and pregnancy history. These observations may be explained by "polyclonal B cell activation".

A number of T-cell abnormalities have been demonstrated in patients with ITP. There are three main mechanisms by which T cells could be involved in thrombocytopenia in patients with ITP. First, significantly increased Th1/Th2 ratios may be related to ongoing immune activation as part of autoimmunity.[29] Second, release of cytokines interferes with megakaryocyte maturation and/or platelet release.[30] Circulating cytokines may also influence the interaction between B and T lymphocytes causing pre-existing B cells to proliferate and produce antiplatelet autoantibodies.[31] Third, some patients with active ITP showed platelet lysis by T cells.[32] These mechanisms may explain a percentage of patients without measurable antiplatelet antibodies in previous reports[15,16] and our study.

The FlowPRA™ test, designed for HLA-specific IgG antibody detection, is a sensitive test, missing the least number of specificities.[33] The FlowPRA™ test has numerous advantages over the conventional lymphocytotoxicity test. Using FlowPRA™, several samples can be processed within a short time using commercially available beads. FlowPRA™ has been used for the detection of HLA antibodies to predict graft rejection in renal transplant recipients.[34] The demerits of the FlowPRA™ test are its high cost and the fact that it cannot differentiate auto- from allo-HLA antibodies.

In conclusion, flow cytometry is a sensitive assay for detecting the presence of platelet antibodies. HLA class I antibodies are frequently found in patients with ITP. Screening for platelet antibodies including HLA antibodies is warranted in patients with ITP. Platelet antibodies including unappreciated HLA class I antibodies may contribute to platelet transfusion refractoriness in patients with ITP.

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